Functional Analysis of a Tryptophan-Less P-glycoprotein: A Tool for Tryptophan Insertion and Fluorescence Spectroscopy

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ABSTRACT

P-glycoprotein (Pgp) functions as an ATP-dependent drug efflux pump to confer multidrug resistance to tumor cells. In the absence of a high-resolution structure for this protein, several important and intriguing aspects of Pgp structure and function remain poorly understood. Fluorescence spectroscopy of endogenous or genetically engineered tryptophan residues represents a potentially powerful method to probe static and dynamic aspects of Pgp at high resolution. We have used site-directed mutagenesis to modify the wild-type (WT) mouse mdr3 Pgp for tryptophan fluorescence spectroscopy by replacement of all 11 tryptophan residues individually with phenylalanine. None of the 11 tryptophans were found to be absolutely essential for Pgp activity, because Chinese hamster ovary cells transfected and overexpressing this mutant Trp-less mdr3 cDNA (mdr3F1–11) become multidrug-resistant and can carry out active transport of vinblastine, colchicine, and Calcein-AM. The mdr3F1–11 mutant has reduced activity compared with WT Mdr3, and shows a unique pattern of drug resistance clearly distinct from WT and, as opposed to the latter, can neither confer FK-506 resistance nor functionally complement ste6 in yeast. Studies with Pgp mutants containing either single or double tryptophan residues or with chimeric molecules constructed between wild-type Pgp and mdr3F1–11 indicated that no single tryptophan residue was responsible for the reduced activity of the mdr3F1–11 mutant. Likewise, all but one chimeric Pgp preserved the unique drug resistance profile of the mdr3F1–11 mutant. Altogether, we show that a Trp-less Pgp is functionally active and can be used as a molecular backbone for insertion of tryptophans in strategic locations to probe various aspects of Pgp function.

Multidrug resistance (MDR) in certain tumors in vivo and in cultured cell lines in vitro is phenotypically defined by cross-resistance to structurally and functionally dissimilar compounds. In many cases, MDR is associated with overexpression of members of the P-glycoprotein (Pgp) family (Hanna and Gros, 1996). These are integral membrane proteins that function as energy-dependent efflux pumps to reduce intracellular drug accumulation. Pgps are encoded by a small family of two closely related genes in humans (MDR1 and MDR2) and three genes in mouse (mdr1, mdr2, and mdr3). In normal tissues, Pgps function as lipid flippases (Ruetz and Gros, 1994) to translocate different types of phospholipids from the inner to outer leaflets of the lipid bilayer in the canalicular membrane of hepatocytes (Schinkel et al., 1994) and possibly in other membranes as well (Vanhelvoort et al., 1996). The mechanism of drug transport by Pgps may be similar to the translocase mechanism of lipid transport demonstrated in normal tissues (Ruetz and Gros, 1994). Pgps belong to the superfamily of ATP-binding cassette transporters, which has been conserved in eukaryotes and prokaryotes (Ling, 1997). Structural homology among ATP-binding cassette transporters translates into functional similarity, as Pgp (Mdr3) (Raymond et al., 1992) can functionally complement null mutations at the yeast ste6 homolog.

Hydropathy profiling (Gros et al., 1986), accessibility to protease cleavage sites (Yoshimura et al., 1989), epitope mapping of inserted antigenic peptides (Kast et al., 1996), photoaffinity labeling (Greenberger, 1993), and modification by sulphydryl reagents (Loo and Clarke, 1995b) have been used to identify structural features of Pgp, and to establish structure/function relationships. Pgp is organized into two symmetrical halves, each consisting of six transmembrane (TM) domains and one nucleotide binding (NB) site. Indepen-

ABBREVIATIONS: MDR, multidrug resistance; Pgp, P-glycoprotein; TM, transmembrane; NB, nucleotide binding; CHO, Chinese hamster ovary; MEM, minimal essential medium; VBL, vinblastine; ADM, Adriamycin; PAGE, polyacrylamide gel electrophoresis; COL, colchicine; ACT, actinomycin-D; VRP, verapamil; VBLR, VBL-resistant; G418R, G418-resistant.
dent lines of investigation have suggested that the TM domains are the primary sites of protein-substrate interaction. These studies include epitope mapping studies of tryptic peptides labeled with drug analogs (Greenberger, 1993) as well as the altered drug-resistance profiles encoded by Pgps bearing naturally occurring (Devine et al., 1992) or experimentally induced mutations in TM domains (Loo and Clarke, 1994; Hanna et al., 1996; Hakemeyer et al., 1998). Pgp has two NB sites of the Walker type that have been positioned intracellularly (Kartner et al., 1985; Yoshimura et al., 1989). Biochemical studies of purified wild-type protein (Senior, 1998; Shapiro and Ling, 1998) and genetic studies with site-directed mutants in the Walker A and B motifs (Azzaria et al., 1989) have shown that ATP binding and hydrolysis at both NB sites are required for Pgp function. Complete cooperativity is required between the two NB sites because mutations at one site abrogate ATP hydrolysis by the protein (Urbatsch et al., 1998). An alternate site catalysis model has been proposed as a mechanism for ATP hydrolysis by Pgp (Senior, 1998). A unique property of its ATPase activity is that it can be strongly stimulated by either substrates or inhibitors of Pgp transport (Senior, 1998; Shapiro and Ling, 1998).

Although significant progress has been made in identifying regions of Pgp involved in drug binding and ATP hydrolysis, important structural and functional aspects of Pgp remain poorly understood. These include identifying the structural determinants responsible for the 1) broad substrate specificity of Pgp, 2) signaling between TM domains and NB sites to mediate activation of ATPase activity and drug efflux, and 3) cooperativity between the two NB sites for ATP hydrolysis. A high-resolution structure of Pgp together with dynamic information will ultimately be needed to answer these questions accurately. In the absence of more detailed structural information, other methods based on site-specific modification with sulfhydryl reagents in single cysteine mutants have been used to monitor changes in the local environment of specific residues and to establish structure/function relationships (Liu and Sharom, 1996; Loo and Clarke, 1997b). Fluorescence spectroscopy of endogenous tryptophan residues (Mdr3F 1–11) as follows: the carboxy-terminal (1.7-kb SphI from polylinker, to SmaI, position 1767) and the amino-terminal (1.7-kb SmaI, position 1767, to PstI, position 3516) half of a mouse mdr3 cDNA cloned in pGEM7Zf were introduced in the corresponding restriction enzyme sites of plasmid vector M13 mp19, as described previously (Kast et al., 1995; Hanna et al., 1996). The W44F (F1), W132F (F2), W158F (F3), W208F (F4), W228F (F5), and W311F (F6) mutations were introduced sequentially on the mdr3 amino-terminal template to generate the mdr3F 1–6 mutant in which the 6 Trp residues from the amino-terminal half of Mdr3 are replaced by the structurally similar Phe. In parallel, the W694F (F7), W704F (F8), W809F (F9), W851F (F10), and W1104F (F11) mutations were introduced in the carboxy-terminal template to generate the mdr3F 7–11 mutant. The two mutated mdr3 halves were then reassembled into a full-length mdr3 cDNA encoding a Pgp mutant devoid of tryptophan residues (Mdr3F 1–11) as follows: the ApII (position 169) to SmaI (position 1767) fragment of M13mdr3F 1–6 was first inserted into the corresponding sites of plasmid pGEM7Zf, followed by insertion of the SmaI (position 1767) to PstI (position 3516) fragment from M13mdr3F 1–11 to create pGEMmdr3F 1–11. Briefly, site-directed mutagenesis was carried out on single stranded DNA template using a commercially available in vitro system (Sculptor system, Amersham Pharmacia Biotech) and mutagenic oligonucleotide primers listed in Table 1. The presence of specific site-directed mutations and the integrity of the rest of the sequence of the mdr3 cDNA inserts used were verified by nucleotide sequencing, before reassembling of the full-length mdr3F 1–11 mutant. For expression in Chinese hamster ovary (CHO) (LR73) cells, the mdr3F 1–11 cDNA from pGEMmdr3F 1–11 was excised as a 4.1-kb KpnIClaI fragment (polylinker sites) and reinserted into the corresponding sites of mammalian vector MluI site.

### Materials and Methods

**Site-Directed Mutagenesis and Plasmid Construction.** To create a Pgp mutant lacking tryptophan residues (F 1–11), the amino-terminal (1.7-kb SphI from polylinker, to SmaI, position 1767) and the carboxy-terminal (1.7-kb SmaI, position 1767, to PstI, position 3516) half of a mouse mdr3 cDNA cloned in pGEM7Zf were introduced in the corresponding restriction enzyme sites of plasmid vector M13 mp19, as described previously (Kast et al., 1995; Hanna et al., 1996). The W44F (F1), W132F (F2), W158F (F3), W208F (F4), W228F (F5), and W311F (F6) mutations were introduced sequentially on the mdr3 amino-terminal template to generate the mdr3F 1–6 mutant in which the 6 Trp residues from the amino-terminal half of Mdr3 are replaced by the structurally similar Phe. In parallel, the W694F (F7), W704F (F8), W809F (F9), W851F (F10), and W1104F (F11) mutations were introduced in the carboxy-terminal template to generate the mdr3F 7–11 mutant. The two mutated mdr3 halves were then reassembled into a full-length mdr3 cDNA encoding a Pgp mutant devoid of tryptophan residues (Mdr3F 1–11) as follows: the ApII (position 169) to SmaI (position 1767) fragment of M13mdr3F 1–6 was first inserted into the corresponding sites of plasmid pGEM7Zf, followed by insertion of the SmaI (position 1767) to PstI (position 3516) fragment from M13mdr3F 1–11 to create pGEMmdr3F 1–11. Briefly, site-directed mutagenesis was carried out on single stranded DNA template using a commercially available in vitro system (Sculptor system, Amersham Pharmacia Biotech) and mutagenic oligonucleotide primers listed in Table 1. The presence of specific site-directed mutations and the integrity of the rest of the sequence of the mdr3 cDNA inserts used were verified by nucleotide sequencing, before reassembling of the full-length mdr3F 1–11 mutant. For expression in Chinese hamster ovary (CHO) (LR73) cells, the mdr3F 1–11 cDNA from pGEMmdr3F 1–11 was excised as a 4.1-kb KpnIClaI fragment (polylinker sites) and reinserted into the corresponding sites of mam-

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**Table 1**

<table>
<thead>
<tr>
<th>Amino Acid Mutant (W → F)</th>
<th>Primer Sequence</th>
<th>5' Nucleotide</th>
</tr>
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<tbody>
<tr>
<td>W44F (F1)</td>
<td>5'-CCCTGCCAGGAAAAACCTGCTAATAC-3'</td>
<td>292</td>
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<tr>
<td>W132F (F2)</td>
<td>5'-CTGCCAGCAAAGAAATTGTAAAGACCTAC-3'</td>
<td>557</td>
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<tr>
<td>W158F (F3)</td>
<td>5'-CACATCAAGAGAACACCTACCTC-3'</td>
<td>634</td>
</tr>
<tr>
<td>W208F (F4)</td>
<td>5'-GGTTAGCTTGAAGCCACGGGT-3'</td>
<td>784</td>
</tr>
<tr>
<td>W228F (F5)</td>
<td>5'-CAATATACCTTTGGAAAATTCCAGCCAC-3'</td>
<td>847</td>
</tr>
<tr>
<td>W311F (F6)</td>
<td>5'-GAAGTCCCTAGAAGAAATGCCCACCGCQTGATGCTAGTACAG-3'</td>
<td>1095*</td>
</tr>
<tr>
<td>W694F (F7) and W704F (F8)</td>
<td>5'-AAAAATTCAAGGGATTCAGTGAAATATCATCAGTGATTCCAGGATCCCGAAAAAGGAACG-3'</td>
<td>2273</td>
</tr>
<tr>
<td>W799F (F9)</td>
<td>5'-GTCACTAAAAAGAGCTCATAC-3'</td>
<td>2557</td>
</tr>
<tr>
<td>W851F (F10)</td>
<td>5'-GTGTTAGTTGGAAGCCCATAGA-3'</td>
<td>2714</td>
</tr>
<tr>
<td>W1104F (F11)</td>
<td>5'-GTGCCTGAGGAAACTGGAACATT-3'</td>
<td>3473</td>
</tr>
</tbody>
</table>

* Additional introduction of the MluI site.
Functional Analysis of a Tryptophan-Less P-glycoprotein

malian expression vector pCB6 (Brewer, 1994). This vector contains both a neo cassette (G418 resistance) for drug selection in transfected cells, as well as a promoter/enhancer region of cytomegalovirus that directs high level transcription of cloned cDNA inserts. For expression in the yeast Saccharomyces cerevisiae and also for further subcloning of various chimeras into pCB6, the mdr3F1-11 fragment, cDNA from pGEMmdr3F1-11, was excised as a 3.3-kb AflI/PstI fragment and inserted into AflI/PstI-digested pVT-mdr3.5 (Urbatsch et al., 1998). The pVT plasmid contains a uRA3 marker for selection in S. cerevisiae and uses the alcohol dehydrogenase gene promoter to direct high level expression of inserted cDNAs (Vernet et al., 1987).

Additional mutants were constructed in which tryptophan residues were re-introduced on the backbone of the tryptophan-less mdr3F1-11 mutant. For this, chimeric molecules consisting of wild-type mdr3 and mdr3F1-11 were constructed. Mutants mdr3F1-14, mdr3F2-7, mdr3F5-9, and mdr3F10-11 were created by inserting segments of pVT-mdr3F1-11 into the corresponding sites of pVT-mdr3F1-14 (625-bp AflI/MscI fragment, positions 169–794), mdr3F2-7 (1454-bp MscI EcoRII fragment, positions 794–2248), and mdr3F5-9 (880-bp SalI/PstI fragment, positions 2630–3510). Mutant mdr3F2-7 was created by recombinant polymerase chain reaction from two overlapping mdr3 products: the first product was generated from plasmid template pGEMmdr3F1-11 using oligonucleotide primers T7 universal (plasmid vector and EC2R, 5′-GGTTAGCTTCCAGCGCGGCTTTATC-3′; positions 759–784), whereas the second was obtained from pVTmdr3 as template with primer pairs EC2F, 5′-GATTACCGCGGGCTTGAAG-3′ (positions 759–778), and CK1, 5′-GATGATCTCCCGAGATGACC-3′ (positions 1099–1119). The 800-bp T7/EC2R and 360-bp EC2F/CK1 products were gel-purified, mixed, denatured, and reamplified using CK1 and T7 universal primer pair. The 1.1-kb fragment product was digested with BglII (position 169) and XhoI (position 794) and reintroduced into the corresponding sites of pVT-mdr3 to create pVT-mdr3F2-7. The 625-bp AflI/MscI fragment was also reintroduced into pVT-mdr3F1-14 to create the single tryptophan mutant, pVT-mdr3W208. The single tryptophan mutant pVT-mdr3W851 and the double mutant pVT-mdr3W208/W851 were created by introducing a 755-bp SalI/XhoI fragment (positions 2630–3385) from pVT-mdr3 into the corresponding sites of pVT-mdr3F1-11, pVT-mdr3W208, and pVT-mdr3W851, respectively. These chimeric and mutant cDNAs in pVT were then introduced as 2215-bp AflI/XhoI (positions 170–3385) fragments into the corresponding sites of either pCB6mdr3 (for pVT-mdr3F1-14), pVT-mdr3F2-7, and pVT-mdr3F5-9) or of pCB6mdr3F10-11 (for pVT-mdr3W208, pVT-mdr3W851, pVT-mdr3W208/W851, and pVT-mdr3F10-11).

**Cell Culture.** Drug-sensitive LR73 CHO cells were grown in minimal essential medium (α-MEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin (50 U/ml) and streptomycin (50 µg/ml). pCB6mdr3 constructs were introduced by transfection into LR73 cells as calcium chloride precipitates, as described previously (Kast et al., 1996). After 2 days, cells were subcultured (1:3 dilution), and stable transfecteds were selected in medium containing G418 (1 mg/ml). Mass populations of G418-resistant (G418-resistant) transfecteds were harvested after 9 days of selection and subcultured in medium containing vinblastine (VBL 25, 50, and 100 ng/ml final concentration) to obtain mass populations of drug-resistant cell clones overexpressing the Mdr3 proteins. VBL-resistant (VBL-resistant) populations were harvested 2 to 3 weeks later, expanded in culture, and frozen at −80°C in 90% serum and 10% dimethyl sulfoxide. In some experiments, individual VBL clones were picked, expanded in culture, and stored frozen until subsequent functional characterization.

**Membrane Preparation and Western Blotting.** Crude membrane fractions from transfected CHO cells were isolated as described previously (Kast et al., 1995). Briefly, cells were grown to 70% confluence and harvested in cold PBS containing sodium citrate. The cell pellet was homogenized (Dounce homogenizer, 50 strokes) in buffer containing 1 mM MgCl2 and 10 mM Tris, pH 7.0, supplemented with protease inhibitors leupeptin (1 µg/ml), pepstatin A (1 µg/ml), aprotinin (1 µg/ml), and phenylmethylsulfonyl fluoride (1 µM). Unbroken cells and nuclei were removed by centrifugation (200g, 5 min, 4°C), and a crude membrane fraction was further isolated by centrifugation of the supernatant (200,000g, 30 min, 4°C). The protein concentration in the crude membrane fraction was determined by the method of Bradford, using a commercially available reagent (Bio-Rad). For immunodetection of Mdr3, 10 µg of protein was resolved on a SDS-7.5% polyacrylamide gel and transferred onto a nitrocellulose membrane by electroblotting. The blots were blocked overnight at 4°C in a solution containing 1% BSA (Fraction V, fatty acid free) in TBST buffer (10 mM Tris, pH 8.0; 150 mM NaCl, 0.05% Tween 20). This was followed by incubation for 1 h with either 1 µg/ml mouse anti-Pgp monoclonal antibody C219 (Centocor Corp., Philadelphia, PA), a 1:400 dilution of mouse anti-human P-gp monoclonal antibody Ab-2 (NeoMarkers, Union City, CA), or a 1:200 dilution of polyclonal-specific rabbit anti-mouse Mdr3 polyclonal antibodies B2037 or K2037 (Devaut and Gros, 1990). Both antisera are raised against the same Mdr3-specific oligopeptide immunogen but coupled to BSA (B2037) or keyhole limpet hemocyanin (K2037). Specific immune complexes were detected using either a second goat anti-mouse antibody (1:10,000 dilution) or anti-rabbit antibody (1:10,000 dilution) coupled to peroxidase and revealed by enhanced chemiluminescence (NEL Life Science Products).

**Cell Cytotoxicity Assay in Mammalian Cells.** Drug cytotoxicity assays were performed using sorfombifurine B to stain cellular proteins, as described previously (Tang-Wai et al., 1993). Briefly, 7.5 × 104 cells were transfected into either VBL-resistant mass populations expressing individual Mdr3 proteins and drug-sensitive control LR73 cells were seeded in 96-well plate containing α-MEM supplemented with increasing concentrations of cytotoxic drugs VBL, colchicine (COL), actinomycin-D (ACT), and Adriamycin (ADM). The cells were incubated at 37°C for 96 h and fixed for 1 h in 10% trichloroacetic acid in PBS, and cellular protein was stained for 10 min at room temperature with 0.4% sorfombifurine B in 1% acetic acid. The plates were washed with water and dried, and the stain was dissolved in 0.2 ml of 10 mM unbuffered Tris. Quantification of sorfombifurine B was done using an automated enzyme-linked immunosorbent assay plate reader (model 450, Bio-Rad) set at a wavelength of 490 nm. The relative plating efficiency of each clone was determined by dividing the absorbance observed at a given drug concentration by the absorbance detected in the same clone in the absence of drug, and is expressed as a percentage. The degree of resistance is calculated by comparing the IC50 value of control cells for a particular drug to the IC50 value of Pgp-expressing cells.

**Drug Transport Assays.** For VBL accumulation, drug-sensitive LR73 control cells and mdr3-transfected clones expressing individual mutant Pgps were grown to confluence and harvested by trypsin treatment. Cells were resuspended in complete α-MEM and allowed to recover for 1 h at 20°C. After this period, cells were centrifuged (300g, 5 min) and resuspended in PBS containing glucose (50 mM) and glutamine (5 mM), at a final cell density of 4 × 10^6 cells/ml. Transport was initiated by the addition of 3H-VBL (specific activity of 0.11Ci/mmol; final concentration 1 µM), and at predetermined time intervals 0.5-ml aliquots of cell suspension were centrifuged through a 200-µl cushion of silicone oil/mineral oil (4:1, v/v). The walls of the tube were washed with 1 ml of PBS, and the pellet was dissolved in 1 N NaOH for a minimum of 16 h. The solution was neutralized by the addition of an equivalent volume of 1 N HCl, and samples were removed for analysis of 3H content and protein concentration. For ADM accumulation experiments, drug-sensitive LR73 control cells and mdr3-transfected clones were harvested by trypsin treatment and seeded in 6-well plate slides (1.2 × 10^6/well) in complete Dulbecco's minimal essential medium (10% fetal calf serum, 0.4% glutamine, and antibiotics). Twenty-four hours later, medium was removed and replaced by Dulbecco's minimal essential medium containing 5% fetal calf serum, 1.0% D-glucose (4:1), and amphotericin B (5 µg/ml), a 1:400 dilution of mouse anti-human P-gp monoclonal antibody Ab-2 (NeoMarkers, Union City, CA), or a 1:200 dilution of polyclonal-specific rabbit anti-mouse Mdr3 polyclonal antibodies B2037 or K2037 (Devaut and Gros, 1990). Both antisera are raised against the same Mdr3-specific oligopeptide immunogen but coupled to BSA (B2037) or keyhole limpet hemocyanin (K2037). Specific immune complexes were detected using either a second goat anti-mouse antibody (1:10,000 dilution) or anti-rabbit antibody (1:10,000 dilution) coupled to peroxidase and revealed by enhanced chemiluminescence (NEL Life Science Products).
tion of 2 μM (specific activity, 2.4 μCi/μmol) with glucose and glutamine. At different times after initiation of transport (incubations at 37°C), cells were washed twice with ice-cold PBS and removed from the well by trypsin treatment. Cells were washed once with PBS, and cell-associated radioactivity was measured directly by adding to liquid scintillation fluid (Cytoscan; Beckman Instruments, Berkeley, CA), followed by vortexing and scintillation counting. A separate aliquot of cells was used for total protein measurement, and the results are expressed as incorporated [14C]ADM/100 μg of protein.

Calcine-AM Transport Assay. Accumulation of Calcine-AM in control LR73 cells and in pCB6mdr3F1–11 transfecteds was measured as described previously (Essodaigui et al., 1998). Briefly, 1 × 10^6 cells from VBL^R mass populations expressing individual mdr3 mutants were resuspended in HPMI medium (120 mM NaCl, 5 mM KCl, 0.4 mM MgCl_2, 0.04 mM CaCl_2, 10 mM HEPES-Na, pH 7.4, 10 mM NaHCO_3, 10 mM glucose, and 5 mM Na_2HPO_4) and incubated in a quartz cuvette with constant agitation. Cells were incubated with Calcine-AM (0.25 μM), and the fluorescence of the intracellular Calcine was continuously monitored for 20 min. After 12 min, the Pgp inhibitor verapamil (VRP; 15 μM) was added to block efflux of Calcine-AM, and thus establish the specificity of the Pgp-mediated reduction in Calcine accumulation in mdr3F1–11 transfectants. Fluorescence was measured at an excitation wavelength of 493 nm and an emission wavelength of 515 nm in a fluorescence spectrophotometer (F-3010, Hitachi Ltd., Tokyo, Japan).

Immunofluorescence. Cells grown on glass coverslips were washed in PBS and then fixed with 4% paraformaldehyde in PBS for 30 min. After three washes in PBS, cells were then permeabilized by treatment with 0.05% NP-40 in PBS with 1% BSA (Fraction V, Roche Molecular Biochemicals, Indianapolis, IN) and 5% normal goat serum (Life Technologies, Inc., Gaithersburg, MD). Coverslips were washed again with PBS and blocked for 1 h at room temperature in PBS containing 1% BSA and 20% normal goat serum. The cells were incubated with the primary antibody (B2037) diluted 1:4000 in blocking solution for 3 h at room temperature, followed by three washes in PBS containing 0.5% BSA and 0.5% Tween 20. Samples were then incubated with secondary antibody for 30 min (Cy3-conjugated goat anti-rabbit IgG, 1:3000, Jackson Immunoresearch, Avondale, PA), followed by three washes in PBS/BSA/Tween 20, and one final wash in PBS. The coverslips were then mounted onto glass slides in ImmuMount (Shandon, Pittsburgh, PA). Immunofluorescence was analyzed with a Zeiss laser scanning confocal microscope using the 63× oil immersion objective and Zeiss LSM software. The same confocal settings were used to analyze all samples.

Purification of Pgp Mutants and Assay for ATPase Activity. The Mdr3^F1–11 and W208 mutants were expressed in the yeast Pichia pastoris after introduction in the plasmid vector pHILD2, according to the protocol described previously (Urbatsch et al., 1998). Positive clones were screened initially by their inability to grow on methanol-containing medium, and were verified, and it was then subcloned in the mammalian expression vector pcB6 (see Materials and Methods) for expression in cultured cells. To assess biological activity of the mdr3^F1–11 mutant, drug-sensitive LR73 CHO cells were transfected with pCB6mdr3F1–11 and mass populations of G418^R colonies were harvested 8 days later. These were then plated in dishes containing increasing concentrations of VBL (25, 50, and 100 ng/ml). At the higher VBL doses (50 and 100 ng/ml), drug-resistant colonies only appeared in cells transfected with wild-type mdr3, whereas no colonies formed in the pCB6mdr3F1–11 group. On the other hand, several VBL^R colonies could be isolated from mass populations of pCB6mdr3F1–11 transfectants in the lower dose of VBL (25 ng/ml). In additional experiments, several of the pCB6mdr3F1–11 VBL^R clones selected at 25 ng/ml were picked and expanded in culture and selected for increasing levels of resistance by subsequent passage in medium containing 50 and 100 ng/ml VBL.

Functional Analysis of mdr3F1–11-Transfected Clones. As a first step for functional analysis of mdr3F1–11 transfecteds, several independent clones isolated in VBL25 and further expanded in VBL100 were expanded in culture and crude membrane fractions were prepared. These membrane fractions were then analyzed by SDS-PAGE and immunoblotting for the presence of the mutant mdr3^F1–11 Pgp. An analysis of three such pairs of clones (clones 3, 4, and 5) is shown in Fig. 2. In these analyses, two anti-Pgp antibodies were used, the mouse monoclonal C219 that recognizes mouse, hamster, and human Pgp isoforms (Kartner et al., 1985) and the rabbit polyclonal B2037 antiserum (Devault and Gros, 1990), which is species- and isoform-specific for the mouse Mdr3 protein. Membrane fractions from untransfected CHO cells and from cells transfected with the wild-type mdr3 cDNA and selected in VBL25 and VBL100 were used as negative and positive controls in these experiments. Clones 3 and 4 express a 150- to 160-kDa protein that is detected by the B2037 anti-mouse Mdr3 antibody. This protein is not detected in untransfected CHO LR73 controls and is of identical electrophoretic mobility as that seen in the control cells transfected with WT mdr3 and selected in the same conditions. This result indicates that the mdr3^F1–11
Pgp is active and confers drug resistance to mammalian cells. Clone 5 is used as an internal control that overexpresses the endogenous hamster Pgp. This hamster Pgp does not react with the anti-mouse Mdr3 B2037 antiserum but instead expresses a C219-reactive protein that is of distinct electrophoretic mobility from the mouse mdr3F1-11 Pgp, and which is expressed at very low level in the parental CHO cells.

The drug resistance spectrum encoded by the mdr3F1-11 Pgp mutant was analyzed in cell cytotoxicity assays for the drugs VBL, COL, ACT, and ADM (Fig. 3). The drug concentration required to reduce the plating efficiency of each mass population by 50% (IC50) was measured and is also expressed as a degree of resistance over CHO controls (Table 2). The results of these analyses were very similar for the two independent mdr3F1-11 Pgp transfectants tested and clearly indicate that the Trp-less mutant encodes a unique drug resistance phenotype distinct from that encoded by either wild-type mouse or hamster Pgps. Indeed, mdr3F1-11 Pgp transfectants selected in VBL25 showed robust VBL (3x, 4x) and COL (4x, 3x) resistance levels that were similar to those seen in CHO transfectants selected in the same conditions and expressing the hamster Pgp (clone 5). By contrast, the mdr3F1-11 Pgp mutant conferred very low levels of resistance to ADM (2.4x, 2.1x) and seemed to retain little if any resistance to ACT (1.2x, 0.8x) when compared with wild-type hamster Pgp (ADM, 3x; ACT, 3x). Adaptation of clones 3 and 4 (mdr3F1-11 Pgp) to growth in VBL100 resulted in a commensurate increase in the level of resistance to this drug (15x, 16x), with final levels of resistance comparable with those seen in CHO transfectants expressing either the wild-type hamster (18x) or mouse Pgp (22x). However, VBL100 selection of clones 3 and 4 caused only a modest increase in resistance to COL (7–9x), ADM (3.3–4x), and in particular ACT (1.8–2x), with final levels of resistance clearly inferior to those measured in cells expressing either wild-type hamster (COL, 26x; ADM, 19x; ACT, 18x) or mouse Pgp (COL, 40x; ADM, 24x; ACT, 50x). These results indicate that the mdr3F1-11 Pgp does indeed confer drug resistance but with a drug resistance profile quantitatively and qualitatively distinct from its parental counterpart.

Next, we analyzed the kinetics of accumulation of Pgp substrates [3H]VBL (cells in suspension) and [14C]ADM

![Fig. 1. Secondary structure model of mouse mdr3 and chimeric constructs. Positions of TM domains, extracellular and intracellular loops, and NB domains are shown, and amino acids are represented by the one-letter code. The native tryptophan (Trp) residues are highlighted as black circles with white lettering. Numbering and positions of the 11 Trp are shown below, followed by the construction of the chimeric Mdr3 molecules. Dark bars represent the wild-type Mdr3 backbone, whereas the white portion consists of the Trp-less backbone. The designation of each chimera is shown to the right.](image-url)
(monolayer cultures) in mdr3F1–11 Pgp (clones 3, 4), wild-type mouse Mdr3 (WTmdr3), or wild-type hamster Pgp (clone 5) transfectants (Fig. 4, A and B). The mdr3F1–11 Pgp transfectants can significantly reduce the accumulation of both drugs over a 30- or 180-min assay when compared with nontransfected cells. In general, cells expressing the mdr3F1–11 Pgp mutant reduced drug accumulation to a level intermediate between that seen in nontransfected controls and that of transfectants expressing either wild-type mouse or hamster Pgps. This was most easily observed when [3H]VBL was used as a substrate and was most obvious for clones selected in VBL25 (low level resistance). We also analyzed the accumulation of another Pgp substrate, Calcein-AM, in these same cell populations (Homolya et al., 1993). Calcein-AM is a nonfluorescent molecule, but once inside the cell, cytoplasmic esterases cleave the acetoxymethyl group and the resulting Calcein molecule becomes fluorescent. In this assay, cells in suspension were incubated in the presence of Calcein-AM and constant monitoring of fluorescence was measured over 12 min. At this point, the Pgp inhibitor VRP was added to the cuvette to block Pgp transport, and fluorescence was further monitored for 8 min (Fig. 4C). In nontransfected CHO cells, we observe a steady rate of accumulation of the fluorescent Calcein molecule inside the cell, and the rate at which fluorescence increases is unaffected when VRP is added. In CHO transfectants, the expression of the mdr3F1–11 Pgp mutant caused a significant reduction in intracellular accumulation of Calcein-AM over that measured in untransfected CHO controls, during the first 12 min of the assay (early part of the trace). This reduction was not as pronounced as that seen for cells expressing either wild-type mouse (WTmdr3) or hamster Pgp (clone 5), but it was also abrogated by the Pgp inhibitor VRP (arrow on graph). Overall, these results show that the mdr3F1–11 Pgp mutant retains biological activity, although at a reduced level when compared with wild-type Pgp. This indicates that tryptophan residues are not essential for Pgp function, although their systematic substitution to phenylalanine causes a partial loss of function.

**Characterization of Mdr3 Chimeras and Single Tryptophan Mutants in Mammalian Cells.** In the next series of experiments, we wished to determine whether the partial loss of function observed in the mdr3F1–11 Pgp mutant was associated with the loss of a specific, particularly important tryptophan residue or the result of an additive effect of several tryptophan replacements. For this, we constructed a series of chimeric Mdr3 molecules in which portions of the wild-type protein were inserted in the backbone of the mdr3F1–11 Pgp mutant to reintroduce different sets of tryptophans. In addition, we reintroduced single tryptophans at positions 208 (W208) and 851 (W851) and at both positions (W208/851) (schematic representation in Fig. 1). The chimeras were cloned into the mammalian expression vector pCB6 and transfected into LR73 CHO cells to assess biological activity. Stable transfectants were selected in G418, and mass populations of G418R colonies were further selected in two different concentrations of VBL (25 and 50 ng/ml). At VBL25, drug-resistant colonies emerged within 2 weeks of selection for cells transfected with either wild-type mdr3 or with mdr3 mutants W208, W851, and W208/851 and chimeras F1–3, F1–4, F5–7, and F10–11. Cells transfected with pCB6 alone yielded no drug-resistant colonies in VBL-containing medium.

Expression of the chimeric and mutant Mdr3 proteins in CHO cells was analyzed by Western blotting. Crude membrane fractions from control drug-sensitive cells and cells expressing wild-type and mutant Pgps were separated on 7.5% SDS-PAGE and analyzed using the anti-Pgp antibody C219 (Fig. 5A). A specific immunoreactive band of approximately 140 kDa was seen in all VBLR mdr3 transfectants, and this band was absent in control nontransfected CHO cells. Although not identical, the expression levels of the various mutants were found to be similar. An immunoreactive band of 110 kDa could also be seen in all the VBLR mdr3 transfectants, in particular in mutants W208/851 and W851. This faster migrating species has been previously suggested to correspond to immature underglycosylated forms of Pgp. The possible functional relevance of this 110-kDa species is unclear, but no correlation between amount of this species and drug resistance phenotype was noted in the various mutants studied. Immunoblotting of the same membrane with the isoform-specific anti-mouse Mdr3 antibody K2037 (Fig. 5A) also confirmed that the immunoreactive band of 140 kDa from the various mdr3 transfectants corresponds to mouse Mdr3 and is not hamster Pgp overexpressed in clone 5 (hPgp). This indicates that the different Mdr3 chimeras and mutants are all capable of conferring resistance to VBL.

The drug resistance profiles exhibited by the various chimeras and mutants were determined for ACT, VBL, COL, and ADM (Table 3, IC50 and fold resistance). Chimeras F1–3, F1–4, F5–7, and F10–11 all conferred a higher level of resistance to the four drugs [ACT (10–47×), VBL (24–59×), COL (10–21×), and ADM (3–15×)] compared with mdr3F1–11 Pgp. On the other hand, the reduced overall activity and altered substrate specificity of mdr3F1–11 (reduced resistance to ACT and COL, robust resistance to VBL) was recapitulated in mdr3 mutants bearing single tryptophans at positions 208 (W208), 851 (W851), or at both positions (W208/851). Finally, all mutants and chimeras, with the exception of F10–11, showed...
severely impaired resistance to ADM, with an almost 10-fold reduction in IC50 value, when compared with our wild-type mdr3 transfectants. This raised the possibility that a targeting defect mislocalizing the protein intracellularly may account for this more severe selective loss of ADM resistance.

We performed confocal microscopy on permeabilized cells expressing wild-type Mdr3 and a subset of cells expressing the chimeras, which included F1–11, W208, W208/851, W851, and F10–11. As shown in Fig. 6, we observed that the staining pattern seen in wild-type Mdr3-expressing cells was identical...
with the cells expressing the chimeric Mdr3 proteins, indicating that the different Mdr3 chimeras are all present at the plasma membrane.

The ability of the Mdr3 chimeras and single tryptophan mutants to transport Calcein-AM also was examined. In all the VBLR clones expressing wild-type Mdr3 and Mdr3 mutants, a significantly decreased accumulation of Calcein in the cytoplasm was observed (Fig. 5B). Furthermore, addition of the Pgp inhibitor VRP resulted in increased accumulation and fluorescence of Calcein at a rate similar to that in non-transfected CHO cells. The levels of Calcein-AM transport in wild-type and mutant Mdr3-expressing cells was similar in all cases, as was the degree of inhibition by VRP. These results verify that all Mdr3 chimeras and mutants are competent for transport and retain susceptibility to inhibition by VRP. Although we did not measure true efflux rates, these results and those shown in Fig. 4 suggest that it is the drug export function of Pgp that is impaired in these mutants. These results suggest that no single tryptophan residue is responsible for the partial loss of function and unique phenotype of the \textit{mdr3} \textit{F}_{1–11} Pgp mutant.

**ATPase Activity of the Mdr3F1–11 and W208 Mutants.**

The Mdr3F1–11 and W208 mutants were expressed in the yeast \textit{P. Pastoris} and purified (Fig. 7A) according to a protocol recently described (Lerner-Marmarosh et al., 1999) and based on dodecyl maltoside extraction of Pgp from microsome-rich fractions, followed by affinity chromatography on Ni$^{2+}$-NTA resin, and final purification by ion exchange chromatography on DE-52 resin (see Materials and Methods). Results from purification experiments (Fig. 7A) show a significant degree of enrichment of wild-type and mutant protein in the imidazole (200 mM) eluate of the Ni$^{2+}$-NTA resin and show excellent purification of the Mdr3F1–11 (1 µg), W208, (2 µg), and WT Mdr3 (5 µg) on subsequent DE-52 chromatography. The purified proteins were reconstituted in the presence of dithiothreitol and \textit{Escherichia coli} lipids and then assayed for ATPase activity. As shown in Fig. 7B, wild-type Pgp and the Mdr3F1–11 and Mdr3W208 mutants displayed low and variable levels of basal ATPase activity; however, ATP hydrolysis by the wild-type protein was greatly enhanced with the addition of 50 µM VRP in agreement with previously published data (Lerner-Marmarosh et al., 1999). In contrast, mutants Mdr3F1–11 and W208 also displayed VRP-induced ATPase activity, although the level of stimulation was smaller than that seen in wild-type Mdr3, with 2.9× and 3.4×, respectively, observed. These results show that removal of all tryptophan residues in the Mdr3F1–11 mutant results in a significant reduction of the drug-inducible ATPase activity of the protein, which correlates with the partial loss of activity of this protein noted in drug resistance and drug transport assays.

**Discussion**

Most of the structural information on Pgp has been gathered from computer-assisted analysis of the predicted amino acid sequence of the protein (hydropathy) (Gros et al., 1986), classical biochemical (mapping of photolabeled proteolytic fragments) (Yoshimura et al., 1989), and immunological analyses (epitope mapping) (Kast et al., 1996) as well as low resolution structure (2.5 nm) by electron microscopy and single particle image analysis (Rosenberg et al., 1997). Additional information has been obtained from the study of single cysteine Pgp mutants constructed on a cysteine-less background, which can be modified by cysteine-reactive sulphydryl reagents. A combination of membrane permeant and impermeant maleimide reagents has been used in various single cysteine mutants to study the topology of individual TM domains of Pgp (Loo and Clarke, 1995b), monitor the activity of individual NB sites (Loo and Clarke, 1995a), and map individual residues contributing to substrate binding (Loo and Clarke, 1996, 1997b). This mutant has also been
used in cross-linking experiments to establish proximity relationships between individual TM domains (Loo and Clarke, 1996, 1997a,b). Finally, cysteine mutants cross-linked with fluorescent maleimide derivatives have been used in fluorescence energy transfer experiments with fluorescent lipid molecules and/or drug substrates to detect structural and/or functional interactions between the membrane domains and the NB sites (Liu and Sharom, 1998).

Fluorescence spectroscopy, which takes advantage of either covalently linked fluorescent reporter molecules or the intrinsic fluorescence of tryptophan or tyrosine residues, is an attractive alternative to study such interactions. In these experiments, intrinsic fluorescence of tryptophan residues can be altered qualitatively (emission wavelength) or quantitatively (emission intensity) by changes in the environment of individual tryptophan residues, including quenching by substrate or inhibitor molecules located in close proximity. This method has been used extensively to identify structure/function relationships in soluble as well as integral membrane proteins. Examples are the study of the catalytic cycle of F1-ATPase (Weber et al., 1998), monitoring dynamic changes in the NB site of the bacterial arsenite extrusion pump during ATP hydrolysis (Zhou and Rosen, 1997), and probing the interaction of lactose analogs with individual membrane domains of the lactose permease of E. coli (Wang et al., 1997). A major limitation of fluorescence studies using endogenous tryptophan residues is the potential complexity of the signal produced by multiple tryptophans in the protein. This can be alleviated by mutational modification to remove all tryptophans from the protein followed by re introduction of single tryptophans at strategic positions in this Trp-less backbone.

To this end, we have constructed a mouse Mdr3 Pgp mutant in which all 11 tryptophans have been changed to phenylalanine (mdr3F1–11). The mdr3F1–11 Pgp mutant is expressed in the membrane fraction of transfected cells, confers drug resistance, and is active in drug transport assays using three known Pgp substrates, Calcein-AM, ADM, and VBL. This result indicates that none of the 11 endogenous Trp residues of Pgp are essential for function. Additional characterization of the mdr3F1–11 mutant indicates a decrease in overall activity when compared with wild-type Pgp, as well as a deviation in the drug resistance profile conveyed in CHO cells when compared with either wild-type mouse or hamster Pgp. The unique drug resistance phenotype of the F1–11 mutant is unlikely to be caused by the selection procedure used, as it was seen in independent cell clones expressing this protein. In addition, we have shown previously that selection of transfected cell clones in low concentrations of VBL has no effect on the drug resistance profile conveyed by wild-type or mutant mdr3 genes.

**TABLE 3**

<p>| Drug survival characteristics of hamster cell clones stably expressing wild-type and mutant mdr3 genes |
|-----------------------------------------------|----------|----------|----------|</p>
<table>
<thead>
<tr>
<th>ACT</th>
<th>VBL</th>
<th>COL</th>
<th>ADM</th>
</tr>
</thead>
<tbody>
<tr>
<td>LR73</td>
<td>5 ± 1 (1×)</td>
<td>10 ± 1 (1×)</td>
<td>47 ± 3 (1×)</td>
</tr>
<tr>
<td>WTmdr3</td>
<td>314 ± 34 (63×)</td>
<td>486 ± 45 (9×)</td>
<td>2560 ± 229 (54×)</td>
</tr>
<tr>
<td>F1–11</td>
<td>5 ± 1 (1×)</td>
<td>163 ± 13 (16×)</td>
<td>553 ± 104 (11×)</td>
</tr>
<tr>
<td>W208</td>
<td>58 ± 6 (12×)</td>
<td>329 ± 45 (33×)</td>
<td>437 ± 26 (9×)</td>
</tr>
<tr>
<td>W208/851</td>
<td>15 ± 3 (8×)</td>
<td>230 ± 10 (23×)</td>
<td>140 ± 60 (5×)</td>
</tr>
<tr>
<td>W581</td>
<td>9 ± 2 (2×)</td>
<td>160 ± 10 (16×)</td>
<td>438 ± 120 (10×)</td>
</tr>
<tr>
<td>F1–3</td>
<td>76 ± 5 (15×)</td>
<td>515 ± 105 (52×)</td>
<td>655 ± 235 (14×)</td>
</tr>
<tr>
<td>F1–4</td>
<td>88 ± 8 (18×)</td>
<td>585 ± 35 (59×)</td>
<td>700 ± 250 (15×)</td>
</tr>
<tr>
<td>F10–11</td>
<td>235 ± 85 (47×)</td>
<td>235 ± 15 (24×)</td>
<td>1010 ± 143 (21×)</td>
</tr>
</tbody>
</table>

*The drug survival of LR73 drug-sensitive cells and multidrug-resistant clones transfected with either wild-type mdr3 (WT) or mutant genes is expressed as the IC50 or the dose necessary to reduce the plating efficiency of the control and transfected cells by 50%. The IC50 value is expressed in ng/ml and is an average (±S.E.) of three independent experiments performed in duplicate.

*The resistance index is calculated by comparing the IC50 value of individual transfecants to that of control LR73 cells and is shown above in parentheses.*
mutant Pgp variants (Beaudet and Gros, 1995). Likewise, we noted that the \textit{mdr3}F\textsubscript{1–11} mutant loses the ability to confer resistance to the fungicide FK506 in yeast and fails to complement a null \textit{ste6} yeast mutant (data not shown), known characteristics of the wild-type mouse Mdr3 protein (Raymond et al., 1992, 1994). Studies of single and double tryptophan mutants and chimeric proteins constructed between wild-type Pgp and \textit{mdr3}F\textsubscript{1–11} indicate that the partial loss of function of \textit{mdr3}F\textsubscript{1–11} is not caused by the loss of any uniquely important Trp residues but is, rather, the result of a cumulative loss of several Trp residues. Studies of the VRP-stimulated ATPase activity of the \textit{mdr3}F\textsubscript{1–11} and W208 mutants indicate that partial loss of drug resistance in these mutants is concomitant to a partial loss of ATPase activity (\textit{F}\textsubscript{1–11}, 2.9× stimulation; W208, 3.4× stimulation). These results support a loss of catalytic activity, as opposed to mistargeting of the mutant enzymes to explain the partial loss of Pgp function observed. Characterization of the drug resistance profiles conveyed by the \textit{mdr3}F\textsubscript{1–11} mutant, and by the additional mutants in which the majority of tryptophans had been substituted by phenylalanine revealed an intriguing low level resistance of all the mutants (with the exception of \textit{F}\textsubscript{10–11}) toward the drug ADM. This finding was surprising, because 1) some of the mutants show near wild type levels of resistance to other drugs and 2) drug transport assays in the \textit{mdr3}F\textsubscript{1–11} mutant for the drug ADM showed a fairly robust transport activity of the mutant toward this drug. The potential causes of this unique phenotype remain unclear. One possibility is that the elimination of one or more tryptophans in Pgp affects protein maturation and targeting. Although this may have little effect on the ability of the corresponding transfectants to prevent drug accumulation in a short-term transport assay, it may nevertheless result in a small increase in steady-state accumulation of ADM only detectable in a longer term cell cytotoxicity assay. This was investigated using confocal microscopy of permeabilized CHO cells expressing the various \textit{mdr3} mutants. There appears to be no major defect in targeting of the protein to the plasma membrane, because all the mutants show a ring-like staining indicative of plasma membrane expression similar to the wild-type \textit{mdr3} protein (Fig. 6).

The \textit{mdr3}F\textsubscript{1–11} mutant constructed here can now be used as a molecular backbone to introduce individual tryptophans at strategic locations in the protein to monitor dynamic changes in local environments as a function of transport or catalytic activity of the protein. Of particular interest are the two single tryptophan mutants already available (W208 and W851) that map, in short extracellular loops of Mdr3 proximal to membrane-spanning domains, regions that have been shown to be involved in drug binding. The effect of different

Fig. 6. Immunofluorescence of chimeric \textit{mdr3} mutants. Untransfected cells (A) or cells expressing (B) wild-type \textit{mdr3}, (C) \textit{mdr3}F\textsubscript{1–11}, (D) \textit{mdr3}W208, (E) \textit{mdr3}W208/851, (F) \textit{mdr3}W851, or (G) \textit{mdr3}F\textsubscript{10–11} were analyzed using immunofluorescence. Cells were fixed, permeabilized, and blocked as described under Materials and Methods followed by exposure to the rabbit anti-mouse Pgp antiserum B2037, then by incubation with a second goat anti-rabbit antibody conjugated to Cy3. Cells were visualized and photographed using a confocal microscope as described previously.

Fig. 7. Purification of Pgp mutants and assay of ATPase activity. A, Coomassie Blue-stained 7.5% SDS-containing polyacrylamide gel of wild-type Mdr3, \textit{Mdr3}F\textsubscript{1–11}, and W208 at different purification steps, including microsomal membranes (15 μg), 200 mM imidazole eluate from an Ni\textsuperscript{2+}-NTA column (5 μg), and flow-through from DE52 column. B, ATPase activity of wild-type Pgp and \textit{Mdr3}F\textsubscript{1–11} and W208 mutants purified from \textit{P. Pastoris}. Purified protein fractions were assayed for ATPase activity in the presence (gray bars) or absence (white bars) of 50 μM VRP.
Pgp substrates and inhibitors on intrinsic fluorescence of these two tryptophans should help investigators understand the nature and complexity of the drug binding site(s) of Pgp. Likewise, the insertion of single tryptophans in either NB site should make possible additional probes of the catalytic cycle of the Pgp ATPase, including understanding dynamic changes at the NB sites associated with drug binding at the TM regions.

References


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